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Direct Measurement of the Poliovirus RNA Polymerase Error Frequency In Vitro

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The fidelity of RNA replication by the poliovirus-RNA-dependent RNA polymerase was examined by copying homopolymeric RNA templates in vitro. The poliovirus RNA polymerase was extensively purified and used to copy poly(A), poly(C), or poly(I) templates with equimolar concentrations of noncomplementary and complementary ribonucleotides. The error frequency was expressed as the amount of a noncomplementary nucleotide incorporated divided by the total amount of complementary and noncomplementary nucleotide incorporated. The polymerase error frequencies were very high and ranged from 7×10^{-4} to 5.4×10^{-3}, depending on the specific reaction conditions. There were no significant differences among the error frequencies obtained with different noncomplementary nucleotide substrates on a given template or between the values determined on two different templates for a specific noncomplementary substrate. The activity of the polymerase on poly(U) and poly(G) was too low to measure error frequencies on these templates. A fivefold increase in the error frequency was observed when the reaction conditions were changed from 3.0 mM Mg^{2+} (pH 7.0) to 7.0 mM Mg^{2+} (pH 8.0). This increase in the error frequency correlates with an eightfold increase in the elongation rate that was observed under the same conditions in a previous study.

Poliovirus RNA replicates in the cytoplasm of infected cells by using a virus-coded-RNA-dependent RNA polymerase. The purified polymerase synthesizes full-length copies of poliovirion RNA and other polyadenylated RNAs in vitro in the presence of an oligo(U) primer (9, 32, 33). Synthetic homopolymers, including poly(A) · oligo(U), poly(C) · oligo(I), poly(I) · oligo(C), and poly(U) · oligo(A) serve as templates and primers for the polymerase.

The rapid evolution of RNA viruses is now a well-documented phenomena (for a review, see reference 16). A low fidelity of replication by viral RNA polymerases could explain in part how this rapid evolution takes place. Many different indirect methods have been used to measure the mutation rates of RNA viruses. These methods have included the measurement of reversion frequencies of viral mutants (12, 15), the measurement of the loss of recognition sites for neutralizing antibodies (20, 23, 26, 27, 34, 35), and the measurement of changes in T_1 oligonucleotide maps (3, 5, 14, 22–24, 29, 31). The only direct measurement of base substitution frequencies has been done with vesicular stomatitis virus (VSV) in which an error frequency of 2.2×10^{-4} was observed in vivo (30).

Studies of the fidelity of poliovirus RNA replication have a unique advantage over studies with most other RNA animal viruses since the viral polymerase has been purified in a soluble and template-dependent form and can be used to directly measure error frequencies in vitro on different RNA templates. By copying synthetic homopolymers with the purified polymerase and differentially labeled complementary and noncomplementary ribonucleotide substrates, one can directly measure the error frequency of the polymerase. This procedure eliminates the bias of converting mutation rates to error rates of the polymerase. The in vitro procedure also has the distinct advantage of allowing one to vary the reaction conditions and observe any changes in the error frequency. Templates may also be varied to determine if certain base pair mismatches are allowed more often than others.

Our findings indicate that the poliovirus polymerase has a very high error frequency of 10^{-3} to 10^{-4}. The error frequency was not significantly affected by using different ribonucleotide substrates and templates. Reaction conditions which are known to correlate with faster elongation rates, however, were found to significantly increase the error frequency.

MATERIALS AND METHODS

Polymerase reaction conditions. The standard reaction mixture (final volume, 30 μl) contained 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 8.0), 3 mM $MgCl_2$, 10 mM dithiothreitol, 2.5 μg of poly(A), 1.25 μg of oligo(U), and 3 μl of fraction 4-HA polymerase purified as described previously (36). Any changes in the standard conditions are indicated in table footnotes and figure legends. For the double-label ^3H-^{32}P misincorporation assays, 10 μCi of [5,6-^{3}H]UTP (36 Ci/mmol) was added as the correct ribonucleoside triphosphate and 20 μCi of either [α-^{32}P]ATP, [α-^{32}P]GTP, or [α-^{32}P]CTP (410 Ci/mmol) was added as the incorrect ribonucleoside triphosphate. Labeled ribonucleoside triphosphates were obtained from Amersham Corp., Arlington Heights, Ill. Unlabeled ribonucleoside triphosphates (Calbiochem-Behring, La Jolla, Calif.) were added to make the reaction mixtures equimolar (7.2 μM) with respect to complementary and noncomplementary ribonucleoside triphosphates. The reactions were run for 1 h at 30°C. The labeled product RNA was precipitated in 7% trichloroacetic acid and 1% sodium PP_i, collected on membrane filters, and counted.

RNA digestion with P1 nuclease. Product RNA was synthesized on a poly(A) template as described above, with 10 μCi each of [^{32}P]UTP and 10 μCi of either [^{32}P]ATP, [^{32}P]GTP, or [^{32}P]CTP. The labeled product RNA was phenol-chloroform extracted, ethanol precipitated, dissolved in 100 μl of 0.1 M NaCl, 1 mM EDTA, 10 mM Tris

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TABLE 1. Error frequency of the poliovirus RNA polymerase

Reaction conditions[a]	Noncomplementary substrate	Complementary substrate	Error frequency[b]
Poly(A) 3 mM $MgCl_2$ pH 8	[^{32}P]ATP [^{32}P]CTP [^{32}P]GTP	[^{3}H]UTP [^{3}H]UTP [^{3}H]UTP	$(3.8 \pm 0.6) \times 10^{-3}$ $(2.9 \pm 0.4 \times 10^{-3}$ $(3.8 \pm 0.5) \times 10^{-3}$
Poly(A) 7 mM $MgCl_2$ pH 8	[^{32}P]ATP [^{32}P]CTP [^{32}P]GTP	[^{3}H]UTP [^{3}H]UTP [^{3}H]UTP	$(5.2 \pm 0.4) \times 10^{-3}$ $(3.4 \pm 0.6) \times 10^{-3}$ $(5.4 \pm 0.3) \times 10^{-3}$
Poly(A) 7 mM $MgCl_2$ pH 7	[^{32}P]ATP [^{32}P]CTP [^{32}P]GTP	[^{3}H]UTP [^{3}H]UTP [^{3}H]UTP	$(1.5 \pm 0.5 \times 10^{-3}$ $(2.5 \pm 0.3) \times 10^{-3}$ $(3.0 \pm 0.6) \times 10^{-3}$
Poly(A) 3 mM $MgCl_2$ pH 7	[^{32}P]ATP [^{32}P]CTP [^{32}P]GTP	[^{3}H]UTP [^{3}H]UTP [^{3}H]UTP	$(1.5 \pm 0.2) \times 10^{-3}$ $(1.0 \pm 0.2) \times 10^{-3}$ $(0.7 \pm 0.3) \times 10^{-3}$
Poly(C) 3 mM $MgCl_2$ pH 8	[^{32}P]ATP [^{32}P]CTP [^{32}P]UTP	[^{3}H]GTP [^{3}H]GTP [^{3}H]GTP	$(2.9 \pm 1.2) \times 10^{-3}$ $(4.8 \pm 2.0) \times 10^{-3}$ $(2.5 \pm 0.2) \times 10^{-3}$
Poly(I) 3 mM $MgCl_2$ pH 8	[^{32}P]ATP [^{32}P]GTP [^{32}P]UTP	[^{3}H]CTP [^{3}H]CTP [^{3}H]CTP	$(2.2 \pm 0.5) \times 10^{-3}$ $(1.6 \pm 0.4) \times 10^{-3}$ $(1.9 \pm 0.6) \times 10^{-3}$

[a] Reaction conditions were as described in Materials and Methods, except the final volume of the poly(A) reactions was 50 μl. In the poly(C) and poly(I) reactions, the final volume was 30 μl and the total nucleotide concentrations were 25.6 and 16.6 μM, respectively.

[b] The error frequency was defined as the picomoles of noncomplementary nucleotide incorporated divided by the total picomoles of nucleotide incorporated into product RNA. For example, 432,802 cpm of [^{3}H]UMP (2.02×10^{4} cpm/pmol) and 8,038 cpm of [^{32}P]AMP (1.33×10^{5} cpm/pmol) were incorporated at pH 8 and 3 mM $MgCl_2$. A counting efficiency for ^{3}H of 0.33 was assumed.

(pH 7.6), and run over a G-50 spun column as previously described (21) to remove unincorporated labeled nucleotides. The labeled RNA was ethanol precipitated and dissolved in 15 μl of 10 mM sodium acetate (pH 6.0) containing 1.5 U of P1 nuclease (Bethesda Research Laboratories, Bethesda, Md.). The sample was then heat denatured at 100°C for 10 min and cooled to 37°C, and another 1.5 U of P1 nuclease in 15 μl of 10 mM sodium acetate (pH 6.0) was added to the sample. The sample was incubated for 1.5 h at 37°C to complete the digestion.

High-voltage ionophoretic separation. Ionophoretic separation of the P1 nuclease-digested product RNA was performed on Whatman 3MM paper at pH 3.5 as previously described (1, 28). ^{32}P-labeled ribonucleoside monophosphates were located by autoradiography, cut out from the paper, and counted in 5 ml of Aquasol-2 scintillation fluid.

RESULTS

Polymerase error frequency. The error frequency of the poliovirus RNA polymerase was determined by measuring the rate at which a noncomplementary ribonucleotide substrate was incorporated into the product RNA with synthetic homopolymeric RNAs as templates. When poly(A) was used as the template, [^{3}H]UTP was used as the complementary substrate and ^{32}P-labeled ATP, GTP, and CTP were used as the noncomplementary substrates. The picomoles of the noncomplementary nucleotide incorporated divided by the total picomoles of nucleotide incorporated into product RNA was defined as the error frequency of the polymerase reaction. The error frequency determined in these reactions was quite high and ranged from 7×10^{-4} to 5.4×10^{-3} (Table 1). No significant difference in the error frequency was observed among the three noncomplementary ribonucleotide substrates. When poly(C) was used as the template, the error frequency ranged from 2.5×10^{-3} to 4.8×10^{-3} (Table 1). Within the experimental error of these experiments, there was again no significant difference between the error frequencies obtained on the poly(A) and the poly(C) templates at the same pH and Mg^{2+} concentration (Table 1). With a poly(I) template, the error frequencies were about the same as those observed on poly(A) and poly(C) (Table 1). It was not possible to determine error frequencies on poly(U) and poly(G) because very low levels of polymerase activity were observed.

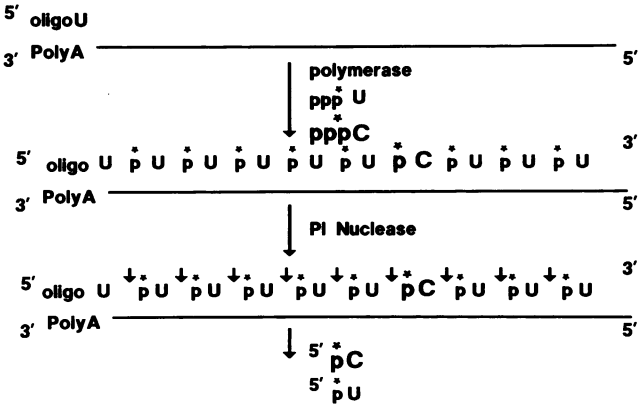

FIG. 1. Diagram showing 5'-ribonucleoside monophosphates recovered from product RNA digested with P1 nuclease. Illustrated is the synthesis of product RNA on a poly(A) template (PolyA) in the presence of an oligo(U) primer (oligoU) by the polymerase in the presence of [^{32}P]UTP (ppp*U) and [^{32}P]CTP (ppp*C). The expected P1 nuclease digestion products that result from cleavage of the labeled product RNA at the sites indicated by the downward arrows are also shown.

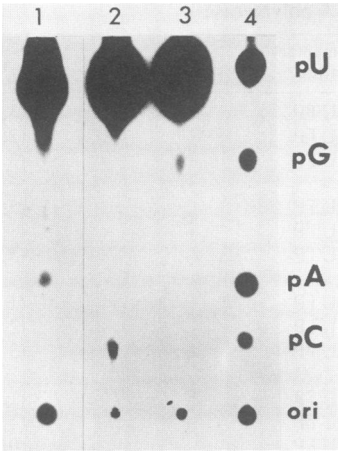

FIG. 2. High-voltage ionophoretic separation of P1 nuclease digestion products. RNA was synthesized in the presence of [^{32}P]UTP and the following ^{32}P-labeled noncomplementary ribonucleotides: [^{32}P]ATP (lane 1), [^{32}P]CTP (lane 2), [^{32}P]GTP (lane 3). Lane 4 is a marker lane containing the ^{32}P-labeled 5′-ribonucleoside monophosphates. UMP (pU), GMP (pG), AMP (pA), and CMP (pC). After digestion with P1 nuclease, the samples were spotted at the origin (ori) and subjected to ionophoretic separation.

P1 nuclease digestion of product RNA. To ensure that the ^{32}P-specific radioactivity incorporated into the product RNA was the noncomplementary nucleotide as opposed to contaminating complementary nucleotide, we analyzed the base composition of the product RNA. Product RNA was synthesized on a poly(A) template with [^{32}P]UTP and a noncomplementary ^{32}P-labeled nucleoside triphosphate, separated from unincorporated labeled ribonucleotides at the end of the reaction, and then digested to completion with P1 nuclease (see Materials and Methods). P1 nuclease digests RNA to 5′-monophosphates and should yield a ^{32}P-labeled noncomplementary ribonucleoside monophosphate in the digestion mixture only if it were actually incorporated into the product RNA (Fig. 1). After digestion with P1 nuclease, the resultant products were separated by high-voltage ionophoretic separation at pH 3.5 on Whatman 3MM paper and located by autoradiography (Fig. 2). When ^{32}P-labeled ATP, GTP, and CTP were used as the noncomplementary substrates, small but detectable amounts of labeled AMP, GMP, and CMP, respectively, were recovered in the digestion products (Fig. 2). To determine the error frequencies, the spots were cut out of the paper and counted. In this case, the error frequencies ranged from 2.0×10^{-3} to 4.8×10^{-3} (Table 2). These values were in agreement with the error frequencies determined under the same reaction conditions by differentially counting the ^{3}H- and ^{32}P-labeled product RNA (Table 1). This indicates that the ^{32}P-labeled nucleotides incorporated in the first assay were not due to small amounts of contaminating ^{32}P-labeled complementary nucleotides. Otherwise, only the incorporation of complementary ribonucleotides would have been observed in the second assay.

TABLE 3. Correlation between polymerase error frequencies and elongation rates

Reaction conditions[a]	Error frequency[b]	Elongation rate[c]
3 mM $MgCl_2$ pH 7	$(0.9 \pm 0.2) \times 10^{-3}$	83
3 mM $MgCl_2$ pH 8	$(3.5 \pm 0.5) \times 10^{-3}$	210
7 mM $MgCl_2$ pH 8	$(4.7 \pm 0.4) \times 10^{-3}$	635

[a] Reaction conditions were as described in Materials and Methods with a poly(A) template.
[b] Each error frequency is the mean of the individual values for the reactions with ATP, CTP, or GTP at each specified reaction condition in Table 1.
[c] Determined under identical reaction conditions with virion RNA as previously described (11).

Error frequencies and elongation rates. Significant changes in the error frequencies were observed when different pHs and Mg^{2+} concentrations were used in the reactions (Table 1). This shows the importance of using the same reaction conditions when comparing the error frequencies with different substrates and templates. In a previous study, we found that the elongation rate of the polymerase reaction also varied significantly with changes in the reaction conditions (11). Increasing the pH from 7.0 to 8.0 and the Mg^{2+} concentration from 3 to 7 mM increased the elongation rate by about eightfold. When the same changes were made in the reaction conditions used in this study, the average error frequency increased about fivefold (Table 3). Thus, there appears to be a direct correlation between an increase in the elongation rate and an increase in the error frequency.

DISCUSSION

In this study, we have examined the fidelity of the poliovirus RNA-dependent RNA polymerase in copying homopolymeric RNA templates in vitro. Highly purified poliovirus RNA polymerase was used to copy poly(A), poly(C), and poly(I) templates in vitro, and the error frequency was expressed as the ratio of the amount of a noncomplementary nucleotide incorporated relative to the total nucleotide incorporated into the product RNA. This general approach has been widely used to measure the fidelity by which purified DNA polymerases copy polynucleotide templates (2, 13). An essential requirement of this assay is that the polymerase be extensively purified and free of any contaminating RNA or terminal transferase. Poliovirus is unique among the RNA

TABLE 2. Polymerase error frequency calculated by digesting ^{32}P-labeled product RNA with P1 nuclease

Template	Noncomplementary substrate	Complementary substrate	Error frequency[a]
Poly(A)	[^{32}P]ATP	[^{32}P]UTP	$(2.9 \pm 2.8) \times 10^{-3}$
3 mM $MgCl_2$	[^{32}P]CTP	[^{32}P]UTP	$(4.8 \pm 2.4) \times 10^{-3}$
pH 8	[^{32}P]GTP	[^{32}P]UTP	$(2.0 \pm 2.4) \times 10^{-3}$

[a] The error frequency was as defined in footnote *b* of Table 1. The picomoles of complementary and noncomplementary substrate incorporated was determined by ionophoretic separations of the ^{32}P-labeled digestion product, locating the monophosphates by autoradiography, and counting them in a scintillation counter (Fig. 2). The values shown are an average of at least three experiments.

animal viruses in that we and others have been successful in purifying the polymerase to near homogeneity in a soluble and template-dependent form (4, 9–11). Essentially no activity above background levels is observed in the absence of exogenously added template and primer RNAs (11).

The results from this study indicate that the poliovirus RNA polymerase has an extremely high error frequency that ranged from 7×10^{-4} to 5.4×10^{-3}, depending on the specific reaction conditions. No significant change in the error frequency was observed when different noncomplementary nucleotides were used in the reactions or when a poly(C) or poly(I) template was used in place of poly(A). In addition, there was no increase in the error frequency for UMP and AMP incorporation on poly(I) although relatively stable I-A and I-U base pairs can theoretically form.

Changes in the reaction conditions were found to result in significant changes in the error frequency. From previous studies, it was known that certain modifications in the reaction conditions would result in about an eightfold increase in the elongation rate of the polymerase reaction. In this study, the same changes resulted in a fivefold increase in the error frequency. These results suggest that a correlation exists between faster elongation rates and higher error frequencies. In future studies, it will be important to determine if other changes in the reaction conditions, such as alterations in the relative nucleotide concentrations and the reaction temperature, and the use of Mn^{+2} in place of Mg^{+2}, significantly affects the error frequency.

The fidelity of the polymerase reaction on synthetic polynucleotide templates may not be exactly the same as that which occurs on RNA. For example, it is possible that some increased misincorporations may be associated with slippage of the primer on the homopolymeric RNA during synthesis of the product RNA. It is very interesting to note, however, that our results are in very good agreement with those of Steinhauer and Holland (30) in which the VSV polymerase error frequency was measured at a single-base site in VSV RNA. The error frequency they observed in vitro ranged from 1.0×10^{-3} to 8.5×10^{-4}, with an average of 7.0×10^{-4}. An average value of 2.2×10^{-4} was observed at the same site for VSV RNA synthesized in vivo. Our results are also in accord with the 10^{-3} to 10^{-4} error frequencies estimated for retrovirus reverse transcriptases (8, 19) and the 10^{-4} error frequency determined for the Qβ RNA polymerase (6, 7).

In a recent study by Parvin et al. (25), the neutral mutation rate in the VP1 gene of poliovirus was assayed by direct sequence analysis. The neutral mutation rate was calculated to be less than 2.1×10^{-6} mutations per nucleotide per infectious cycle. This result is in sharp contrast to the very high polymerase error frequencies obtained in this study and with the VSV polymerase (30). The difference between the low neutral mutation rate measured as described above and the high error frequency determined in vitro raises several questions. Does the viral polymerase exhibit a much higher level of fidelity during RNA replication in vivo or is there a high probability that most mutations are deleterious and are selected against during viral replication?

In future studies, we plan to measure the poliovirus RNA polymerase error frequency during the replication of poliovirus RNA in vivo. These results should help distinguish between two possible mechanisms for the evolution of picornaviruses. If the viral polymerase exhibits a relatively low error frequency during RNA replication in vivo, then a large number of infectious cycles would be required to generate changes in the viral genome. In contrast, a high polymerase error frequency would generate mutations in the genomic RNA after a relatively small number of infectious cycles. It is clear that poliovirus and other picornaviruses rapidly change as they replicate in their natural hosts (5, 17, 18, 23, 24, 31). Determining the polymerase error frequency in vivo, however, should help distinguish between these two mechanisms.

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